A FLAGELLIN-POXVIRUS ANTIGEN VACCINE:
STRENGTHS AND LIMITATIONS

BY

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I hear the horror stories of graduate students all over the country who bemoan the fact that their advisors do not care about their projects, or do not care about their students. I’m incredibly fortunate to have had an advisor who always had my best interests at heart. When you pushed me, I knew it was because you had faith in me and what I could become. I take that as an incredible compliment, and I thank you for believing in me in times that I struggled to believe in myself.

This project ended up in a place so different from what we expected and I feel like I gained more from my graduate experience as a result. It was a journey full of twists and turns, and I’m glad that I had the privilege to learn from you along the way. Thank you.

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For being the person who always listened to my questions and was never too busy to hear my ideas, this thesis is dedicated to you.
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>Avertin</td>
<td>2,2,2, tribromoethanol</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Baggs Albino C</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone Marrow-derived Dendritic Cell</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokind (C-C motif) Ligand</td>
</tr>
<tr>
<td>CVF</td>
<td>Cobra Venom Factor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CRP</td>
<td>C-reactive Protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EEV</td>
<td>Extracellular Enveloped Virion</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GR-1</td>
<td>Lymphocyte antigen 6 complex, locus G</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>eYFP</td>
<td>enhanced Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>F4/80</td>
<td>EGF-like module containing mucin-like hormone receptor-like sequence 1</td>
</tr>
<tr>
<td>FB</td>
<td>Flagellin-B5R</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IgH</td>
<td>Immunoglobulin Heavy Chain</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IMV</td>
<td>Intracellular Mature Virion</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IPTG</td>
<td>Isopropylthiogalactopyranoside</td>
</tr>
<tr>
<td>LF</td>
<td>L1R-Flagellin</td>
</tr>
<tr>
<td>LFB</td>
<td>L1R-Flagellin-B5R</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Ly-6G</td>
<td>Lymphocyte antigen 6 complex, locus G</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemoattractant Protein</td>
</tr>
<tr>
<td>MIP1</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MTD_{50}</td>
<td>Maximally Tolerated Dose for 50% of subjects</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified Vaccinia Ankara</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation primary response gene 88</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>pH</td>
<td>Potential for Hydrogen</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>Th</td>
<td>T Helper Cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia Virus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>WR</td>
<td>Western Reserve</td>
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ABSTRACT

Delaney, Kristen N.

A FLAGELLIN-POXVIRUS ANTIGEN VACCINE:
STRENGTHS AND LIMITATIONS

Dissertation under the direction of

Steven B. Mizel, Ph.D., Professor of Microbiology and Immunology

Bacterial flagellin is a potent adjuvant that enhances adaptive immune responses to a variety of antigens. The vaccinia virus antigens L1R and B5R are highly immunogenic in the context of the parent virus, but recombinant forms of the proteins are only weakly immunogenic. Therefore, we evaluated the response to these antigens when flagellin was used as an adjuvant. Although flagellin promoted a robust antigen-specific humoral response to poxvirus antigens delivered intranasally (i.n.) or intramuscularly (i.m.), intramuscular immunization resulted in significantly high titers of anti-L1R and B5R IgG. Flagellin/poxvirus antigen fusion proteins were more potent than flagellin and L1R and B5R as separate proteins as inducers of a humoral response against the poxvirus antigens. At least three immunizations with flagellin/poxvirus fusion proteins were required to confer protection in mice against challenge with vaccinia virus. Although mice were protected and exhibited only limited signs of disease, they still exhibited significant, but reversible weight loss. When immune mice were depleted of complement using cobra venom factor, 50% of the mice succumbed to vaccinia virus infection. These results demonstrate that flagellin-poxvirus antigen fusion proteins are effective in eliciting protective immunity against vaccinia virus that is dependent, in part, on complement.
We evaluated the efficacy of additional flagellin-poxvirus antigen constructs to promote protective immunity and found that antigens can lose their immunogenicity when inserted into certain regions of flagellin. The loss of immunogenicity is dependent on the individual antigen, and was not the same for all antigens tested. When administering more than one immunogenic fusion protein antigen-specific titers decrease slightly, and the addition of excess flagellin will further decrease titers suggesting that there is a limit to the number of fusion proteins that can be administered in a single vaccine.
INTRODUCTION

Variola Virus Pathogenesis and Life Cycle

The orthopoxvirus Variola virus is a very large double-stranded DNA virus and is the etiological agent of smallpox. The disease was eradicated in 1979 by the efforts of the World Health Organization’s aggressive vaccination campaign (35). Smallpox is spread through respiratory transmission and is associated with respiratory infection and the appearance of “pocks,” confined lesions at sites of concentrated viral replication. In humans, mortality rates can be as high as 30% in some age groups.

Since laboratory use of variola virus is not permitted, vaccinia virus is used as a model agent in poxvirus vaccine studies. Intranasal administration of vaccinia virus leads to systemic infection via the bloodstream 12 hours post-inoculation in rabbits (111) and lasts for more than 7 days. In the murine model, vaccinia virus causes respiratory infection that peaks around day 7 without the appearance of pocks.

Vaccinia Virus Life Cycle and Morphogenesis

Vaccinia virus replication within a host cell begins with viral entry. While the nature of vaccinia virus receptors remain unknown, it is clear that the different infectious forms of the virus enter by different methods (103) and that the extracellular enveloped virion (EEV) enters cells more quickly than intracellular mature virions (IMV) (28). Once in the cytoplasm, early mRNAs are transcribed within the virus core and translated by host machinery. This leads to further uncoating of the core, release of viral DNA, and transcription of later viral genes (92). Approximately 2-5 hours post-infection intracellular mature virions have been assembled. Most of these infectious IMV particles
will remain intracellular until the cell lyses. The bulky virus particles move very slowly through the host cell’s cytoplasm (95), so the virus particles polymerize actin to propel themselves through the cell (21,39). Some of the particles will be pushed through the golgi and acquire a golgi-derived double membrane, these are referred to as intracellular enveloped virus (IEV). These IEV particles translocate to the cell surface where the outermost golgi-derived membrane fuses to the plasma membrane releasing the second infectious form of vaccinia virus, extracellular enveloped virus (EEV).

The IMV form of the virus is very resilient and is therefore implicated in host-to-host spread. The EEV form of the virus is much more fragile, but is more involved in spread throughout a host. EEV is released before cell lysis of the host cell, it enters cells more rapidly than IMV (28), and it has a higher specific infectivity (12.7 virus particles/pfu compared to 64.6 virus particles/pfu for purified IMV) (102). The outer EEV membrane is easily disrupted by mechanical shearing, drying, or low pH. But, loss of the outer membrane yields a hearty IMV particle, so the virion remains infectious. It has been shown that acellular vaccine strategies that target both infectious forms of vaccinia virus provide enhanced protection to challenge than strategies that only use antigens from one infectious form (36,37,49,50,65).

**Poxvirus protective antigens B5R and L1R**

B5R has 2 forms; a 42 kDa transmembrane form, and a 35 kDa secreted form. The protein is N-glycosylated and expressed early and late in infection (32,55). The transmembrane form of the protein is found on the EEV form of the virus, and is the major target of EEV-neutralizing antibodies (8,13,40,59). The secreted form plays a role in evasion of host immune responses by acting as a decoy for B5R-specific antibodies,
thus reducing the amount of B5R-specific antibody available to bind virus-associated B5R. B5R is very important in the pathogenesis of vaccinia virus. Mutant viruses lacking B5R have a 5-10 fold reduction in the formation of EEV particles, form small viral plaques, and are attenuated in vivo (33,67,99,107).

L1R, on the other hand is associated with the IMV particle. It is a 25 kDa protein expressed late in infection. L1R is a major target for antibody-mediated neutralization of IMV (42,54,65). While the actual biological function of L1R is unknown, it obviously plays a critical role in viral development. A mutant virus was developed that placed L1R expression under the control of IPTG. In the absence of IPTG, plaque formation was abrogated and only immature virions could be detected by electron microscopy (86).

Vaccinia Virus Evasion of Host Responses

Vaccinia virus has many gene products dedicated to evasion of the host immune response. Some of the more commonly targeted systems are mentioned here. For full reviews see (43,91).

Interference with Cytokines and Interferons. Many vaccinia proteins are molecular mimics of cytokine receptors. These viral gene products do not have a transmembrane signaling domain, thus they compete with the native receptors for binding with these immune effector proteins. Some examples of poxvirus proteins which provide this function are A53R which mimics the TNF receptor (90), B15R which encodes an IL-1β receptor mimic (2), and B8R which resembles the IFN-γ receptor (101).

Another way that poxviruses interfere with IFN signaling is by preventing detection of virus within infected cells. The E3L gene product binds dsRNA to block the
activation of PKR (24,58) which is a major viral detection method. PKR is also the target of K3L, which prevents its auto-phosphorylation (24,25).

*Inhibition of Apoptosis.* E3L also slows the progression of apoptosis in host cells (24,58) which allows the virus a longer time to assemble before lysis. It is unknown whether this delay is through interaction with PKR, or if this is the result of an interaction with another cellular protein.

*Blockade of TLR Signaling.* The poxvirus protein A52R inhibits TLR-mediated activation of NFκB (14). It associates with IRAK2 and TRAF6 which results in disruption of signaling complexes involving these proteins (44).

**Vaccination Approaches to Providing Protection Against Smallpox**

The current smallpox vaccine approved for use in the United States, Dryvax, is a live strain of vaccinia virus which is administered by scarification. The vaccine has the risk of causing serious complications such as progressive vaccinia, eczema vaccinatum, myopericarditis and ischemic heart disease. Other countries approved the use of other, more reactogenic strains of vaccinia Lister and Coppenhagan which appear to be associated with higher incidence of cardiac complications (85). In the US, vaccination with Dryvax is limited to highly-screened military personnel, health care professionals, and laboratory workers. The vaccine cannot be administered to individuals who are immunocompromised, elderly, very young, or those with atopic dermatitis as they are at high risk for complications. In light of the restrictions on vaccination, there has been a push to develop a safer vaccine that could be used to protect the general populace in the event of malicious release of variola virus.
Alternative vaccination strategies under development include attenuated strains of vaccinia virus, recombinant DNA vaccines, and recombinant protein vaccines. The most well-characterized of these is an attenuated live vaccine; Modified Vaccinia Ankara (MVA). This virus is replication deficient in mammalian cells due to extensive passage in chick embryo fibroblasts and the loss of approximately 15% of the parental genome (74,81). MVA was actually used for widespread immunization in Germany during the WHO’s smallpox eradication campaign (93).

Both the innate and adaptive immune systems are involved in clearance of vaccinia virus in an immunocompetent individual. Two studies evaluated the requirement for different branches of the adaptive system in a Modified Vaccinia Ankara immunization model. Utilizing immunodeficient mice (109) or cell depletion techniques (9) these studies showed that either a T cell response or a humoral response is sufficient to provide protection. Mice only succumbed to infection when both cellular and humoral responses were lost.

**Recombinant Vaccinia Virus Proteins are “Weakly-Immunogenic” Antigens**

Vaccinia virus is a well-studied virus which induces robust humoral and cellular immune responses (for review see (73)). However, when the dominant immunogens of vaccinia virus are removed from the context of the parent virus they show reduced immunogenicity (30,36,37,46,49-51).

DNA vaccination with pox antigens A27L, L1R, A33R, and B5R yields higher antibody titers than scarification with vaccinia virus in mice and non-human primates (30,49). However, when DNA vaccinated macaques (4 vaccinations delivered by gene gun) are challenged with monkeypox they still develop lesions (46). The fact that these
vaccines are less effective in protecting non-human primates than mice suggests they may also provide limited protection in humans.

Recombinant protein vaccines are safer than those involving live viruses, but efficacy remains an issue. In order to obtain complete protection from challenge, mice must be immunized multiple times with recombinant proteins (36,37). For example, Fogg et al. demonstrated that relatively large amounts of protein and at least four immunizations are required to achieve protection against respiratory challenge with vaccinia virus in mice (37). In this study, the authors used trehalose dicorynomycolate emulsion or saponin as adjuvants to enhance the response to L1R, B5R and A33R. These results provided a strong rationale for evaluating other adjuvants that might have a more potent effect on the response to the poxvirus antigens.

Work from our laboratory (48,75,105) as well as other investigators (11,20,52,53,61,62,69,70) has established that flagellin, the major structural protein of Gram-negative flagella, is a potent adjuvant when administered with an antigen by any of several different routes. The adjuvant effect of flagellin has demonstrated in a variety of pathogen models (26,48,52,56,62,69,97,98,104,105).

Flagellin—Structure and Biological Activities

Flagellin Structure. Flagellin is the major structural subunit of bacterial flagella. It consists of 4 major domains—a highly conserved region which is involved in binding and a hypervariable region that differs greatly between various Gram-negative bacterial species. The structure of the protein was originally determined by Yonekura, et. al. (112). The N and C termini are both involved in flagellar polymerization (112). The majority of antibodies generated towards flagellin target the hypervariable region (82);
likely because that is the region that is exposed when flagellin is polymerized. The interaction of flagellin with toll-like receptor 5 (TLR5) is mediated by highly-conserved region in domain 1 (94).

**Flagellin and Pathogenesis.** Flagellin is the major structural subunit of bacterial flagella which are involved in motility of bacteria. Flagellar mutants show reduced virulence in *Pseudomonas* (34,88), and *Salmonella* (16,84,96). Although the loss of flagella reduces pathogenesis, the activation of innate immunity is often markedly reduced. For this reason, the expression of flagella must be tightly regulated to avoid activating the innate immune system. Down-regulation of flagellar gene products reduced the release of inflammatory mediators (IL-8 and GM-CSF) and could provide a selective advantage for *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients (19).

**Flagellin Signals via Toll-like Receptor 5.** Several studies (41,45,77) demonstrated that flagellin signals via toll-like receptor 5 (TLR5). Amino acids 386–407 of flagellin bind to a leucine-rich repeat of TLR5 (78) and induce downstream signaling in a MyD88-dependent manner (45). The binding of flagellin to TLR5 induces IRAK-1 activation (80) and downstream de-repression of the transcription factor NFκB, allowing for transcription of NFκB-responsive genes. This signaling leads to activation of innate immune responses such as production of cytokines (4,5,27,72,80), influx of neutrophils into lungs (47,63), and NO production in macrophages (76).

**Flagellin as an Adjuvant**

**Flagellin’s effects on innate immunity.** Work from the laboratory of Dr. Ruth Arnon provided the first clear evidence that flagellin possesses adjuvant activity (62,70).
Influenza epitopes cloned into flagellin and administered to mice with no other adjuvant induced a protective response against influenza virus. Subsequent studies by a number of groups established that the adjuvant effect of flagellin is due to at least three important actions: 1) induction of cytokine production by non-lymphoid cells (4,5,20,27,72,79); 2) increased T and B lymphocyte accumulation in draining lymph nodes (6); and 3) activation of \( tlr5^{+/+} \) \( CD11c^+ \) cells (7).

1. **Cytokine Production.** Many cytokines are produced by non-lymphoid cells following exposure to flagellin (4,5,20,27,72,79). APCs produce TNF-\( \alpha \) (20,72), IL-8 (5,72) and NO (20) which is indicative of a proinflammatory response. Additionally, many chemokines are produced upon exposure to flagellin including MCP (CCL2) (72) which recruits monocytes, T cells, and DC (15,110). The neutrophil recruiters and activators (108) MIP1a (CCL3), MIP1b (CCL4), are also produced (47,72).

It is also worth noting that flagellin treatment drives the production of IL-4 and IL-13 (5,22,27), which are associated with the classical Th2 allergic response. However, flagellin does not promote the production of IgE (48).

2. **Increased Cellularity.** Twenty-four hours after i.n. instillation of flagellin there is a marked increase in cellularity of draining lymph nodes (6). The majority of the increase was in T and B lymphocytes. It is possible that this was either by increased influx or reduced egress. Either of these possibilities increases the likelihood that a T or B cell will come in contact with an APC which is presenting a recognized antigen.

3. **Flagellin and Dendritic Cells.** In vitro studies with isolated human dendritic cells (1,72,87) clearly demonstrate that flagellin activates dendritic cells (DC). Means, et. al. demonstrated that flagellin treatment of peripheral blood mononuclear cells
(PBMC) results in an upregulation of costimulatory molecules, cytokine production, and a decrease in phagocytosis (72). Additionally, flagellin-treated human DC induced proliferation of CD3\(^+\) T cells (72). There have been varied reports on TLR5 expression in murine DC. Several groups have reported that TLR5 is expressed on bone marrow-derived DC (3,23,31,64), splenic DC (27,31,64), and lamina propria DC (100). The complication with amplifying TLR5 mRNA is that the TLR5 gene does not contain any introns. Therefore, unless a DNase is included in the PCR reaction, it is possible that genomic TLR5 is being amplified. Means et. al. did not detect an effect of flagellin on murine DCs (72). Didierlaurent et. al. observed TLR5 expression in splenic DC, but not bone marrow-derived DC (BMDC). However, when they treated BMDC with flagellin, there was a greater upregulation of costimulatory molecules in BMDC than in splenic DC (27). It is possible that discrepancies within the literature are due to the purity of the flagellin used in the individual studies.

Bates et al. (7) found that purified recombinant flagellin has only a weak effect on cytokine production in cultures of murine BMDC. However, a requirement for CD11c\(^+\) TLR5\(^+\) cells for the adjuvant effect of flagellin on antigen-specific CD4 T cell proliferation was clearly demonstrated (7). Thus it is quite likely that in contrast to lymph node DC, the majority of BMDC are at a stage of development during which TLR5 expression or signaling is extremely limited.

**Flagellin fusion proteins.** In regards to adaptive immunity, it is well-established that flagellin induces the proliferation of antigen-specific CD4\(^+\) T cells (7,71) and robust humoral immunity (4,10-12,20,26,52,53,61,62,69,70,75,89,98,105). There is even some evidence that flagellin can promote CD8\(^+\) T cell proliferation (52). But the effects of
flagellin on adaptive immunity are enhanced when administered in the form of flagellin-antigen fusion proteins (refs: *Yersinia pestis* (48,75), *Pseudomonas aeruginosa* (105), influenza, (10-12,53,56,62,70) and West Nile Virus (69)). Fusion proteins containing flagellin and target antigen are almost ten-fold more potent (7). The ability of flagellin to bind with high affinity to TLR5 (106) on CD11c\(^+\) antigen-presenting cells (APC) is likely to facilitate enhanced uptake of the associated antigen—a process that may ultimately result in a higher level of antigen presentation. (7,48,75).

In light of the potent adjuvant activity of flagellin, we explored the possibility that this adjuvant may be able to dramatically improve the humoral response to the weakly immunogenic vaccinia virus antigens, L1R and B5R. We evaluated this possibility in the context of separate proteins as well as flagellin fusion proteins. The results of these studies have not only established the ability of flagellin to enhance responses to L1R and B5R, but have also illuminated important strengths and weaknesses of flagellin as an adjuvant.
MATERIALS AND METHODS

Mice

Female BALB/c mice (6-8 weeks) were purchased from Charles River Laboratories and were housed in specific pathogen free facilities. IgH\(^{-/-}\) breeders were purchased from Taconic Laboratories. Only female IgH\(^{-/-}\) offspring were used in immunization studies. All animal work was done in accordance with protocols approved by Wake Forest University’s Animal Care and Use Committee.

Cell Lines

RAW264.7 and HeLa cell lines were purchased from ATCC. RAW424 cells were generated by transfecting RAW264.7 cells with an expression plasmid containing TLR5 linked to eYFP (106). Plasmid expression is maintained by the addition of 400 \(\mu\)g/mL G-418 to culture medium.

Purification of anti-CD8 Monoclonal Antibody

TIB-210 cells which produce Rat anti-mouse CD8 IgG (clone 2.43) were purchased from ATCC. Large volumes of cells at a cell density of 5\(\times\)10\(^5\) cells/mL were prepared. The non-adherent cells were removed from the medium by spinning at 2,000 rpm, and the media was retained. All following steps take place at 4°C. Media was concentrated in a large-scale concentrator using a 10kDa cutoff membrane until ~100mL remained. This was then dialyzed overnight into binding buffer (20mM Sodium Phosphate, pH 7.0). The sample was purified on a Protein G HP column (GE Healthcare).
and eluted with 100mM glycine-HCl, pH 2.7. Fraction tubes contained appropriate amounts of 1M Tris-HCl ph 9.0 to bring the pH of the eluates to 7.0. Antibody is dialyzed overnight into PBS for long term storage at -80°C.

**Virus Production and Purification**

Vaccinia Virus Western Reserve (VV-WR) was produced by infecting HeLa cells at an MOI of 5 for 48 hours. Cells were pelleted by centrifugation and lysed by at least 4 freeze-thaw cycles. Further lysis was performed by 3 cycles of sonication at full power for 30 seconds. Virus was centrifuged 80 minutes at 32,900 x g and 4°C on a 36% sucrose cushion in 10mM Tris-Cl, pH 9.0. The pellet was resuspended in 1mM Tris-Cl pH 9.0.

**Baculovirus production of L1R and B5R**

Baculoviruses encoding ectodomains of L1R and B5R were obtained from J.C. Whitbeck, Schools of Dental and Veterinary Medicine, University of Pennsylvania, Philadelphia, PA. Viral proteins were prepared by infecting SF9 cells (Invitrogen) grown in SF-900II Serum Free Medium (Gibco) at an MOI of 3-10. Supernates were harvested on day 3 and concentrated using a filter with a 10kDa cutoff (Millipore). The his-tagged proteins were purified using a Ni-NTA Superflow column (Qiagen). Proteins were then dialyzed into PBS pH 7.0 (L1R) or PBS pH 5.9 (B5R). To remove endotoxin and DNA contaminations, the proteins were run through Acrodisc units with Mustang E (B5R) or Q (L1R) filters (Pall Corporation). Endotoxin levels were <20pg/μg of protein (limulus amoebocyte assay (Cape Cod Inc.)).
Generation of flagellin and flagellin-poxvirus fusion proteins

FliC (hereafter referred to as flagellin) from Salmonella enterica serovar Enteritidis (68) was prepared as previously described (47,68). The 229 truncation mutant of flagellin contains only amino acids 297 – 471 of the hypervariable region, and therefore does not signal via TLR5 (47,68,78) Two approaches were used to generate fusion proteins containing flagellin and L1R or B5R or flagellin, L1R, and B5R. Plasmids containing L1R and B5R cDNA were kindly provided by J.W. Hooper (USAMRIID, Ft. Detrick, MD). To generate a flagellin-B5R (FB) fusion, a cDNA encoding the ectodomain of B5R (defined as nucleotides 57–837) was cloned into the fliC gene lacking the majority of the hypervariable region (lacking nucleotides 586-1134). The L1R-flagellin (LF) fusion protein was generated by inserting the cDNA encoding the ectodomain of L1R (nucleotides 1-543) at the N-terminus of full-length fliC. These constructs are illustrated in Figure 1. Each construct was cloned into the pET29a expression vector (Novagen).

Purification of proteins

Plasmids were transformed into E. coli BL-21 (DE3) for protein production. All proteins used in this study contained a 6-His tag to facilitate rapid purification using Talon metal affinity resin (Clontech) as previously described. To remove endotoxin and nucleic acid contaminants, the purified proteins were passed through Acrodisc Mustang Q filters (Pall Corporation). LPS levels (measured by Limulus Amoebocyte Assay, Cape Cod Inc.) were <20pg/µg of protein.
Figure 1 Proteins used in this study and their TLR5-stimulating activity. Chimeric proteins were produced containing the ectodomains of L1R and B5R fused to flagellin. Diagrams illustrate antigen placement. L1R is fused to the N-terminus of full length flagellin. B5R substitutes the hypervariable domain of flagellin. TLR5-stimulating activity is calculated as units/mg. A unit is the inverse of the concentration yielding a half maximal response. This is then standardized to units/mg.
TLR5 Stimulatory Activity (units/mg)

- 1.9x10^{17}
- 2.1x10^{17}
- 1.4x10^{18}
**TLR5-specific signaling activity of flagellin and flagellin fusion proteins**

In vitro TNFα production was used as a measure of flagellin signaling activity (17,18,47). To insure that the signaling was TLR5 specific, we assessed activity in cultures of RAW 264.7 cells, a mouse macrophage cell line which does not express TLR5 and in cultures of RAW 424 cells, RAW 264.7 cells that stably express TLR5. These cell lines were stimulated for 4 hours with flagellin-containing proteins and supernatants were harvested for analysis of TNF by ELISA using a commercial kit (BD Biosciences) as per the manufacturer’s instructions. Units are calculated as the inverse of the concentration giving a 50% maximal response, which are then standardized to units per milligram.

**Immunizations**

For intranasal (i.n.) immunizations, mice were anesthetized with Avertin (2,2,2-tribromoethanol (Sigma)) and 10-15 μL of vaccine mixture was administered dropwise to alternating nares. For intramuscular (i.m.) immunizations, groups of at least 7 mice were anesthetized with Avertin and then 20 μl of vaccine mixture was injected into the right rear calf. Groups of 7 mice received 2 immunizations on days 0 and 28, 3 immunizations on days 0, 28, and 42, or 4 immunizations on days 0, 28, 42, and 56. Blood samples were collected via tail vein bleeding 10-14 days after each immunization and the plasma prepared for analysis of antibody titers.

**Determination of plasma IgG titers by ELISA**

ELISA plate wells (Immunosorb 96-well plates, NUNC) were coated with 100μL of 10μg/mL recombinant antigen (L1R or B5R) in PBS overnight at 4°C. Plates were
blocked with PBS + 10% newborn calf serum, and then plasma samples were added to triplicate wells. The plates were incubated overnight at 4°C, washed, and then rabbit anti-mouse IgG conjugated to horseradish peroxidase was added and the plates incubated for 2 hours at room temperature, followed by a 30-minute incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate. The reaction was stopped by the addition of 2N H₂SO₄, and absorbances were read at 450nm. Titers were defined as the inverse dilution of plasma which yields an absorbance of 0.1 over background (established using naïve plasma).

**Respiratory challenge with vaccinia virus**

The 50% maximally tolerated dose (MTD₅₀; equivalent to LD₅₀) for BALB/c mice was determined for each vaccinia virus preparation using non-linear regression. Groups of 7 mice were anesthetized with avertin and 10μL of virus was administered dropwise to alternating nares. Mice were observed daily for signs of disease. Symptoms were scored according to the following disease index: Hunched Posture (no = 0, yes = 1), respiratory stress (no = 0, mild-moderate = 1, severe = 2). Conjunctivitis and lethargy were evaluated on the same scale as respiratory stress. A mouse which received a disease score of 2 for any symptom or lost 30% of its initial weight was sacrificed.

**Vaccinia Virus Neutralization**

A flow cytometry-based assay was used to assess vaccinia virus neutralization (29). An eGFP-expressing vaccinia virus was diluted in DMEM with various dilutions of heat-inactivated mouse plasma. HeLa cells (seeded in 96-well plates at 1x10⁵ cells/well)
were infected at an MOI of 0.25 and incubated for 6 hours. The cells were then treated with trypsin and fixed with PFA before FACs analysis. Virus incubated in medium only was used as a control (0% neutralization).

**Depletion of CD8⁺ T cells**

Mice were treated with 0.3mg of monoclonal anti-CD8 antibody (clone 2.43) injected i.p. three times on days -5, -3, and -1 in relation to challenge.

**Complement Depletion**

Mice were treated with 5μg (1 unit) of Cobra Venom Factor (CVF) (Calbiochem) i.v. 18 hours before challenge with vaccinia virus. A second treatment of 5μg of CVF is administered 4 days after the initial injection (3 days post-infection). In preliminary experiments, we established that this protocol depletes complement levels for 5 days with complement levels returning to ~50% on day 6.

**Determination of Circulating Numbers of Neutrophils**

Whole blood was collected in heparinized tubes 1 day after mice had been immunized two or three times. After transfer to FACs staining tubes, a uniform number of FluoSphere microspheres (Invitrogen F8836) were added to the tubes to allow for later standardization of cell numbers. Samples were stained for F4/80 (monocytes; Caltag Laboratories MF48020) and GR-1 (neutrophils; eBioscience 51-5931-80).
**Measurement of C-reactive Protein**

Plasma samples were collected from mice 1 day after mice had been immunized two or three times. Plasma was prepared as described above and stored at -80°C until analysis. C-reactive protein was measured by an ELISA kit (Kamiya Biomedical KT-095) as per the manufacturer’s instructions. Plasma was diluted 1:5 instead of the recommended 1:10.

**Statistical analysis**

Statistical analyses were performed using Prism 5 (GraphPad). Mouse titer data was assumed to be non-parametric, thus the Mann-Whitney Test was utilized to evaluate differences in titers. Survival data was evaluated using the integrated survival analysis tool.
CHAPTER I

GENERATING RESPONSES TO WEAKLY IMMUNOGENIC PROTEINS

USING FLAGELLIN AS AN ADJUVANT
RESULTS

CHAPTER I: Generating Responses to Weakly Immunogenic Proteins Using Flagellin as an Adjuvant

*Immunization with flagellin and pox antigens results in a robust humoral response and protection against respiratory challenge with vaccinia virus.* A number of studies using recombinant L1R and B5R proteins (36,37) or DNA encoding these proteins (46,49-51) have demonstrated that recombinant forms of these antigens are only weakly immunogenic. To evaluate whether flagellin could promote a more robust response to these antigens, mice were immunized i.n. on 2, 3, or 4 occasions with 10 μg of purified Baculovirus-expressed L1R and B5R plus 1 μg of flagellin and assessed for plasma titers of anti-L1R and B5R IgG 10-14 days after each immunization. As shown in Figures 2A and B, the IgG titers against L1R and B5R increased with increasing numbers of immunizations. Although a significant number of mice responded after two immunizations, additional immunizations were required to achieve high titers in most of the animals. In the case of the anti-L1R response, all of the mice responded after 4 immunizations. However, even with 4 immunizations, 4/10 mice did not exhibit significant titers of anti-B5R IgG. These results are in striking contrast to the response to flagellin + the *Y. pestis* F1 and V antigens (6,48,75). In the case of the pestis antigens, two immunizations results in IgG titers that are generally ≥ 5 x 10⁵.

To evaluate the protection conferred by i.n. vaccination, the mice which had received 0, 2, 3, or 4 immunizations were challenged i.n. with 10 MTD₅₀ (equivalent to 10 LD₅₀) of vaccinia virus. Immunizations were staggered so that all mice were challenged at the same time. Mice losing 30% of their initial weight were sacrificed. All
**Figure 2** Intrasanal immunization with pox antigens + flagellin confers protection after 3 or more immunizations. Groups of 10 BALB/c mice were intranasally immunized with 10μg of L1R + 10μg of B5R + 1μg of Flagellin 2-4 times and plasma was collected after each immunization. Titers of antibodies specific for pox antigens B5R (a) and L1R (b) were determined by ELISA. Each symbol represents an individual mouse, and the dashed line at 10³ is the limit of detection for the assay. Numbers below the dashed line indicate the number of mice whose titers were below the limit of detection (non-responders). A single asterisk indicates that the titer is significantly higher than the titer following a single immunization. Other groups which are significantly different are indicated by bars and multiple asterisks. (c) Three weeks after the final immunization, mice were intranasally challenged with 20 MTD₅₀ of vaccinia virus and survival was documented. (d) As an estimate of morbidity associated with challenge, weights were recorded daily following infection.
of the mice that received 3-4 immunizations survived challenge (Fig. 2C), whereas only about 50% of the animals that received 2 immunizations survived. Assessment of weight loss revealed that even the mice that received 4 immunizations lost a significant amount of weight (approximately 15%). However, these mice did not exhibit any other severe signs of disease (for example, difficulty in breathing, conjunctivitis, or hunched posture).

*Protective effect of a flagellin-adjuvanted poxvirus vaccine is dependent on B cells, but not on CD8\(^+\) T cells.* To evaluate which branches of adaptive immunity are required for flagellin-mediated protection against poxvirus infection IgH\(^{-/-}\) mice were immunized three times i.n. with 10\(\mu\)g each of L1R and B5R and 1\(\mu\)g of flagellin. IgH\(^{-/-}\) mice lack the immunoglobulin heavy chain gene, and therefore cannot rearrange a stable B cell receptor. Therefore, the mice do not develop mature B cells. As seen in figure 3, when these mice were challenged with 10 MTD\(_{50}\) vaccinia virus all of them succumbed to infection. We conclude from this that the humoral response to the flagellin + pox antigen vaccine is absolutely required for the protective effect of the vaccine.

To evaluate the role of CD8\(^+\) T cells, BALB/c mice were immunized as described above. Beginning five days prior to challenge, these mice were injected with a depleting rat anti-mouse CD8 IgG antibody (clone 2.43). Mice were injected i.p. with 0.3mg on days -5, -3, and -1 relative to challenge. Figure 4a shows the depletion that results from this anti-CD8 IgG treatment, and figure 4b shows survival after challenge. There was no difference in survival of mice which were treated with the CD8-depleting antibody. Therefore, CD8\(^+\) T cells are not required for the protective effect of the flagellin-based vaccine. However, this experiment does not rule out the possibility that CD8\(^+\) T cells may still contribute to survival.
Figure 3  *Flagellin-mediated protection requires B cells.* BALB/c or IgH⁻/⁻ mice were immunized 3 times i.n. with 10μg each of L1R and B5R and 1μg of flagellin. Three weeks after the final immunization mice were challenged with 10 MTD₅₀ vaccina virus WR. Survival was monitored for two weeks.
Figure 4  *Flagellin-mediated protection does not require CD8\(^+\) T cells.* Groups of 5 BALB/c mice were immunized 3 times i.n. with 10\(\mu\)g of L1R and B5R plus 1\(\mu\)g of flagellin. One group of mice was depleted of CD8\(^+\) cells by depletion with an anti-CD8 antibody (clone 2.43) starting 5 days before challenge. Depleted mice were injected i.p. with 0.3mg of antibody on days -5, -3, and -1 relative to challenge. (A) Depletion of CD8\(^+\) cells in lymph node (LN) and spleen (S). Numbers indicate the percentage of recovered cells within a given quadrant. (B) Survival following i.n. challenge with 10 MTD\(_{50}\) VV-WR.
A

PBS

α-CD8

LN

CD3

S

CD8

B

Percent survival

0 2 4 6 8 10 12 14

Days post-infection

Naive

Untreated

α-CD8

28
Enhanced immunogenicity of flagellin-poxvirus fusion proteins. Several recent studies have demonstrated that flagellin fusion proteins enhance responses to strong antigens such as influenza virus epitopes (10,12,53,56,62,70), the F1 and V antigens of *Y. pestis* (48,75), and West Nile Virus’s EIII (69). Since these fusion proteins promoted a more robust response than separate antigen(s) plus flagellin, we tested whether the immunogenicity of recombinant L1R and B5R could be enhanced by genetically linking them to flagellin. Schematics for the flagellin/pox antigen fusion proteins are shown in Figure 1. To determine if the fusion proteins retained the ability to signal via TLR5, we assessed their ability to stimulate TNFα production in cultures of TLR5+ RAW 424 cells. To control for TLR5-independent signaling, we also assayed TNFα production in cultures of TLR5-negative RAW 264.7 cells. As shown in Figure 1, the LF and the FB retained full TLR5 signaling activity relative to flagellin.

Having established that the fusion proteins retained TLR5 signaling activity, we next compared the ability of the fusion proteins with that of the separate proteins (flagellin + L1R +B5R) to induce IgG responses against L1R and B5R. Groups of 7 mice were immunized i.n. or i.m. with equimolar doses of L1R (1.3μg) + B5R (2.4μg) + flagellin (3.7μg), or LF (5μg) + FB (5μg). As shown in Figure 5A and 5B, the fusion proteins LF and FB elicited IgG that recognized recombinant L1R and B5R. This response was dramatically more robust than the response to separate proteins + flagellin administered i.m. This difference was most evident with the B5R response. Furthermore, the variability in response within the fusion protein groups was far less than that observed when separate proteins were used for immunization.
**Figure 5** *Fusion proteins are more effective at promoting antigen-specific humoral responses than separate proteins.* Groups of 7 BALB/c mice were immunized 2 times i.m. with 5μg of flagellin-antigen fusion proteins LF and FB or equimolar doses of the separate proteins. B5R (A) and L1R (B) specific titers are shown as determined by ELISA. Asterisks indicate significant differences in titer (P < 0.05).
Comparison of i.n. and i.m. administration of flagellin-poxvirus fusion proteins.

With highly immunogenic antigens such as the F1 and V antigens of *Y. pestis*, there is not a dramatic difference in the induced antibody titers when administered with flagellin i.n. vs. i.m. (48). To evaluate the effect of route of administration on the humoral response to the significantly less immunogenic recombinant L1R and B5R, groups of 7 mice were immunized three times (i.n. or i.m.) with LF and FB and evaluated for plasma anti-L1R and B5R IgG titers. As shown in Figure 6A and 6B, immunization via the i.m. route elicited significantly higher titers of anti-L1R and B5R IgG than obtained using the i.n. route of administration. The circulating titers of anti-L1R and B5R in mice immunized i.m. were about two logs higher than those in mice immunized i.m.. Furthermore, the variability in the response was more limited when the i.m. route was used. Thus, the response to recombinant L1R and B5R can be markedly increased by using flagellin fusions and by using the i.m. route of administration.

*B5R-specific IgG2a titer correlates with protection from vaccinia virus challenge.*

A recent study (13) found that passive immunization with a B5R-specific monoclonal antibody confers protection against vaccinia virus challenge. To assess whether IgG2a titer correlated with protection we tested archived plasma samples from mice which had been immunized various times with separate proteins plus flagellin and seroconverted in response to immunization. The plasma samples were taken before challenge, and mice were grouped according to survival following vaccinia virus challenge. Pooled plasma from convalescent mice was used as a positive control. There was no significant difference between survivors and non-survivors in L1R-specific IgG2a titer (Fig. 7). However, the anti-B5R IgG2a titers were significantly lower in mice that were not
**Figure 6** *Intramuscular immunization is more effective at promoting antigen-specific humoral responses than intranasal immunization.* Groups of 7 BALB/c mice were immunized 2 times i.m. or i.n. with 5μg of flagellin-antigen fusion proteins LF and FB. B5R (A) and L1R (B) specific titers are shown as determined by ELISA. Asterisks indicate significant differences in titer (P < 0.05).
Figure 7  Mice who are protected during challenge have significantly higher antigen-specific IgG2a titers than mice who succumb to challenge. Archived plasma samples from immunized BALB/c mice were collected prior to challenge. Samples were separated into mice who went on to survive challenge, or those who succumbed to infection. Pooled plasma from convalescent mice was used as a positive control. Significant differences (P < 0.05) are indicated by asterisks.
protected (Fig. 7A). These findings are consistent with the hypothesis that IgG2a plays a role in antibody-mediated protection against vaccinia virus.

*Immunization with LF and FB promotes the production of antigen-specific IgG1 and IgG2a.* The previous experiment used plasma samples from mice immunized with L1R + B5R + flagellin. To evaluate whether fusion proteins can promote IgG2a titers comparable to those seen in survivors immunized with separate proteins, we immunized mice 2, 3, or 4 times i.m. with 5 μg each of LF and FB (Figure 8). Total IgG, IgG1, and IgG2a antibody titers were determined by ELISA. Total anti-L1R and B5R IgG titers as well as IgG1 and IgG2a increased with increasing numbers of immunization. Similarly, the number of responding mice also increased with each additional immunization. To determine if a higher dose of LF and FB would alter the balance of antigen-specific IgG1 and IgG2a, BALB/c mice were given 3 immunizations with 20 μg of LF and FB (Figure 9). Although the variability in response was markedly reduced, the IgG1 and IgG2a titers were not significantly different in mice which were given 20 μg of the fusions compared to 5 μg.

*Immunization with LF and FB confers protection against respiratory challenge with vaccinia virus.* To determine if fusion proteins decrease the number of immunizations required for protection against vaccinia virus, mice were immunized two, three, or four times i.m. with 5 μg each of LF and FB and then challenged i.n. with 20 MTD50 vaccinia virus. As shown in Figure 10, despite significantly higher poxvirus specific antibody titers, fusion protein vaccination still required at least three immunizations to achieve 100% protection. Although mice given three or four immunizations survived challenge, they still lost a significant amount of weight (Fig.
Figure 8  *IgG, IgG1, and IgG2a responses following i.m. immunization with 5μg of LF and FB.* Mice were immunized i.m. with 5μg of LF and 5μg of FB. (a-b) Total IgG titers, (c-d) IgG1 titers, and (e-f) IgG 2a titers after each immunization were determined by ELISA. Each symbol represents an individual mouse, and the dashed line at 10^3 is the limit of detection for the assay. Numbers below the dashed line indicate the number of mice whose titers were below the limit of detection (non-responders). In (a-d) all groups are significantly different unless noted by n.s. (e-f) significant differences are indicated by asterisks.
**Figure 9** *IgG, IgG1, and IgG2a responses following i.m. immunization with 20μg of LF and FB.* Mice were immunized i.m. with 20μg of LF and 5μg of FB. (a-b) Total IgG titers, (c-d) IgG1 titers, and (e-f) IgG 2a titers after each immunization were determined by ELISA. Each symbol represents an individual mouse, and the dashed line at 10^3 is the limit of detection for the assay. Numbers below the dashed line indicate the number of mice whose titers were below the limit of detection (non-responders). In (a-d) all groups are significantly different unless noted by n.s. (e-f) significant differences are indicated by asterisks.
Figure 10  *Challenge after immunization with 5μg of LF and FB.* Groups of 10 BALB/c mice who received 2-4 i.m. immunizations of 5μg of LF and FB were challenged with 20 MTD$_{50}$ vaccinia virus. (a) Survival, (b) weight loss, and (c) disease index were measured following infection.
Figure 11  Challenge after immunization with 20μg of LF and FB. Groups of 10 BALB/c mice who received 3 i.m. immunizations of LF and FB at the indicated doses were challenged with 20 MTD$_{50}$ vaccinia virus. (a) Survival, (b) weight loss, and (c) disease index were measured following infection.
To evaluate disease symptoms other than weight loss, we used a disease index score that evaluated hunched posture, respiratory distress, conjunctivitis, and lethargy. As shown in Figure 10C, mice that received 3 or 4 immunizations exhibited only minimal disease symptoms. In contrast, mice that were immunized only twice had disease symptom scores that approached those of animals that were not immunized. Since 40% of the mice immunized twice with the fusion proteins did not produce anti-B5R IgG2a, it was possible that the lower level of survival might be associated with this subgroup of animals. However, analysis of individual animals did not reveal a correlation between anti-B5R IgG2a titer and survival (data not shown). It seems likely that the very high titers of anti-B5R IgG1 obtained following immunization with the fusion proteins i.m. (as opposed to separate proteins and i.n. immunization (Fig. 7)) may compensate for the absence of IgG2a in some of the mice.

Complement plays a role in the protective effect of LF + FB. Since IgG2a is known to be an efficient activator of complement in mice and monoclonal anti-B5R IgG2a protects against vaccinia virus challenge (13), we evaluated whether complement-mediated immunity was critical to the protective response generated in response to LF and BF immunization. BALB/c mice were immunized 3 times i.m. with 5μg each of LF and FB. One group of immunized mice was depleted of complement by i.v. administration of CVF 18 hr before challenge with 20 MTD_{50} of vaccinia virus. A second treatment of CVF occurred on day 3 post-infection. In preliminary experiments, we established that two treatments with CVF dramatically reduces the level of circulating C3. Six days after treatment, the level of C3 is only about 50% of that in untreated mice (data not shown). All of the mice that were immunized, but not treated with CVF
Figure 12  *Complement plays a role in the protective effect of a flagellin-poxvirus antigen vaccine.* BALB/c mice were immunized 3 times i.m. with LF and FB. Six mice were treated with cobra venom factor (CVF) on days -1 and 3 relative to challenge with 20 MTD$_{50}$ vaccinia virus. (a) Survival, (b) weight loss, and (c) disease index were measured following infection.
survived infection. However, 50% of the immunized mice that were treated with CVF succumbed to infection (Fig. 12A). Although the degree of weight loss between the two groups was similar (Fig. 12B), the complement-depleted mice had higher disease scores (Fig. 12C). These results are consistent with the conclusion that complement component C3 plays a role in LF + BF-induced protection.

*Virus neutralization titers of plasma from immunized mice.* To evaluate the virus neutralization titer of antibodies generated by immunization with LF + FB, in vitro vaccinia virus neutralization was performed using pooled heat-inactivated plasma collected from mice prior to challenge. Plasma from immunized mice was incubated with eGFP-expressing vaccinia virus for 1 hour prior to incubation with HeLa cells for 6 hours. Virus neutralization was defined as a 50% reduction in eGFP expression compared to virus that was not incubated with plasma. All samples from immunized mice neutralized significantly more virus than plasma from mock-immunized animals (Fig. 13). There was a significant (~3-fold) difference in neutralization between two and three immunizations with 5μg of the fusion proteins LF and FB. Mice immunized by a single exposure to vaccinia virus (convalescent) had titers that were two-fold higher than any of the LF + FB immunized mice. It is interesting to note that the neutralizing titers correlated with the severity of disease. Naïve mice all succumbed to infection and had neutralizing titers under 100. Mice immunized twice were partially protected, lost weight, and had elevated disease scores and had an average neutralization titer of approximately 300. The mice that received 3 or 4 immunizations were protected and had low disease scores, but lost significant weight. Their average neutralizing titers were approximately 900. The mice that received a sublethal dose of vaccinia virus exhibited
Figure 13 *Plasma from immunized mice is capable of neutralizing vaccinia virus in vitro.* Pooled plasma from mice receiving 2-4 immunizations with 5μg of LF and FB or 3 immunizations with 20μg of LF and FB was incubated with a GFP-expressing vaccinia virus for at 37°C at the indicated dilution. The plasma-treated virus was added to HeLa cells and allowed to infect for 6 hours. Infected cells were detected by flow cytometry and percent of neutralization was calculated by standardizing to mock infected cells (100%) or cells infected with virus that was not treated with plasma (0% neutralization). Neutralizing titer is defined as the dilution at which 50% of virus is neutralized, and is calculated by non-linear regression. A reference line is provided at 50% neutralization, and calculated values for neutralization titer are in the legend.
complete immunity and did not exhibit any disease symptoms or weight loss. These mice had titers ≥2,000.

*Analysis of inflammatory effectors following immunization.* Although the changes in neutralizing titer may account for the difference in protection observed between two and three immunizations with LF + FB, one or more additional factors may be involved. We evaluated the possibility that three immunizations with LF + BF might trigger the production of liver-derived C-reactive protein, an acute phase reactant with complement-activating and opsonic activities, and thus compensate for insufficient levels of IgG2a. To evaluate this possibility, mice were immunized i.m. 2 or 3 times with 5μg of LF and FB or PBS, and plasma samples were collected 24 hours after the final immunization. Plasma C-reactive protein levels were determined by ELISA. There was no change in circulating CRP levels of mice immunized with the flagellin fusion proteins compared to PBS-injected mice (Fig. 14A).

We also considered the possibility that after three immunizations, flagellin might induce sufficient titers of GM-CSF (19) that would increase the numbers of circulating neutrophils and monocytes available for opsonization of antibody-coated virions. To evaluate this possibility, we immunized mice as described for the analysis of C-reactive protein levels and collected whole blood after 2 and 3 immunizations with LF and FB. Cells were stained for the markers F4/80 (monocyte marker) and GR-1 (neutrophil marker) and analyzed by flow cytometry. The addition of a known number of fluorescent microspheres allowed us to back-calculate numbers of cells per mL of blood. Monocyte counts remained constant in all tested conditions (Fig. 14B). The drop in neutrophil levels 24 hours after a second immunization with LF and FB is consistent with
Figure 14  Effect of repeated immunization on circulating levels of neutrophils, monocytes, and C-reactive protein. BALB/c mice were immunized two or three times i.m. with 5μg each of LF and FB. (A) Plasma samples were collected 24 hours after the final immunization and analyzed for C-reactive protein levels by ELISA. (B) Twenty-four hours post-immunization whole blood samples were taken and stained for neutrophils (GR-1$^+$, F4/80$^{-lo}$) and monocytes/macrophages (GR-1$^-$, F4/80$^+$). Addition of a known number of fluorescent microspheres allowed for the quantitation of neutrophils and monocytes per mL of blood.
neutrophils being recruited to the site of injection (6,48). After a third immunization with LF and FB, the level of neutrophils in the blood approached that of control mice. Since neutrophilic recruitment to the site of injection would occur after the third immunization, it is likely that the increased level of circulating neutrophils in this group is due to enhanced bone marrow production of these cells. However, additional studies are clearly required to formally test this hypothesis.
CHAPTER II

LESSONS ABOUT FLAGELLIN AS AN ADJUVANT

AND A VACCINE VECTOR
RESULTS

CHAPTER II: Lessons about Flagellin as an Adjuvant and a Vaccine Vector

The constructs used in chapter one were not the only constructs generated in this project, and with these other proteins shown in figure 15 we learned some valuable lessons about the strengths and weaknesses of flagellin. In the currently published studies using flagellin as an adjuvant the target antigens are strong immunogens such as F1 and V from Yersinia pestis (48,75). In model systems with strong immunogens the limitations of flagellin are masked by the inherent immunogenicity of the antigen. These are the limitations we discovered using weakly immunogenic poxvirus antigens.

Variability among antigens for optimal placement within a flagellin fusion protein. The proteins generated in figure 15 were evaluated for their ability to induce antigen-specific humoral responses. BALB/c mice were immunized three times i.n. with the indicated protein. Fourteen days after the final immunization, plasma samples were collected and tested for antigen-specific IgG. FB and LF induce robust antigen-specific humoral responses. However, FL, with L1R replacing the hypervariable region of flagellin, was a very poor inducer of L1R-specific IgG in immunized mice. From this it is evident that individual antigens are limited in the locations they can be inserted into flagellin, but each antigen will be different. This certainly is a limitation of flagellin, but since there are 3 locations (N-terminus, C-terminus, and hypervariable region) in flagellin capable of inserting proteins without disrupting bioactivity it is likely that a site can be found for most antigens that will not compromise immunogenicity.
Figure 15: *Flagellin-pox antigen fusion proteins and their TLR5-stimulating activity.*

Chimeric proteins were produced containing the ectodomains of L1R and B5R fused to flagellin. Diagrams illustrate antigen placement. TLR5-stimulating activity is calculated as units/mg. A unit is the inverse of the concentration yielding a half maximal response. This is then standardized to units/mg.
TLR5 Stimulatory Activity (units/mg)

2.8x10^17

3.8x10^17

3.1x10^17

2.4x10^17
**Figure 16** *Variability among antigens for optimal placement within a flagellin fusion protein*. BALB/c mice were immunized three times i.n. with 5μg each of FB, LF, or FL. B5R (A) and L1R (B) titers were determined by ELISA. The dashed line represents the limit of detection for the assay, and the number below that line indicates the number of mice whose antigen-specific titers were below the limit of detection.
Antigenic competition is observed when LF and FB administered together.

Groups of 7 BALB/c mice were immunized intramuscularly on three occasions with 5μg of both LF and FB or only a single fusion protein. Plasma samples were assayed for B5R and L1R-specific IgG titers. As shown in Figure 17, the combination vaccine induced titers of anti-L1R and B5R IgG that were significantly less than the titers using only a single fusion protein.

We considered two possible explanations for this effect. It was possible that L1R and B5R were involved in some form of antigenic competition and thus the combination of the two would result in a reduced response to both antigens. Alternatively, the reduced response might be due in some way to an inhibitory effect of the flagellin component of each fusion protein. However, based on our prior results (47), we know that flagellin doses up to at least 15 μg do not result in a diminished humoral response to an immunogen.

The humoral response to a flagellin-antigen fusion protein can be reduced by addition of excess flagellin. Thus we considered the possibility that the flagellin components of each fusion were competing for a limiting amount of TLR5 to facilitate efficient uptake of each fusion protein. If this were the case, then co-administration of LF or FB with increasing doses of flagellin should result in a reduced response to the poxvirus antigen. To test this notion, groups of mice were immunized with FB alone or FB with increasing doses of flagellin and then the induced titers of anti-B5R IgG were measured. The results (Figure 18) clearly demonstrate that as the dose of flagellin was increased, the resultant titer of anti- B5R was significantly reduced.
Figure 17  Competition between antigens when multiple flagellin fusion proteins are administered. BALB/c mice were immunized 3 times i.m. with 5μg each of LF, FB, or LF and FB. B5R (A) and L1R (B) titers were determined by ELISA.
**Figure 18** The humoral response to a flagellin-antigen fusion protein can be reduced by addition of excess flagellin. Groups of 7 BALB/c mice were immunized 3 times i.n. with FB alone or FB with excess soluble flagellin. Ratios on the x-axis indicate the FB:Flagellin ratio. Asterisks indicate significant differences.
Although this finding is not a direct proof, it is consistent with the hypothesis that competition is due to a rate-limiting level of TLR5 on antigen-presenting cells.

*A trifusion protein that combines flagellin with two poxvirus antigens loses immunogenicity.* In view of the competition between fusion proteins and the need to generate responses against IMV and EEV antigens, we generated a trifusion protein that contained L1R at the amino terminus of flagellin and B5R as a replacement for the majority of the hypervariable region of flagellin (Fig. 15). This protein retained full TLR5 signaling activity as measured in the RAW cell assay for TNFα production. As seen in figure 19, when groups of mice were immunized with the trifusion protein, the anti-L1R IgG responses were similar to those obtained with L1R-flagellin (mean titer = 5.8x10^5). However, there was no detectable B5R-specific response.

*B5R is not recognized by B5R-specific antibodies in the context of the LFB trifusion.* To determine if the absence of B5R-specific IgG was due to a change in the conformation or availability of the B5R in the trifusion, we coated ELISA plates with LFB and used plasma from mice immunized with B5R and alum as a source of anti-B5R IgG. The B5R-specific plasma did not react with trifusion (Figure 20), indicating that the conformation of B5R is altered in the trifusion, or that the B5R is not accessible to antibodies. Thus, although flagellin exhibits substantial plasticity with regard to the insertion of antigens, the antigens themselves can place limitations on the carrier function of flagellin (as opposed to its adjuvant activity).
Figure 19 *A trifusion protein that combines flagellin with two poxvirus antigens loses immunogenicity.* Groups of 7 BALB/c mice were immunized three times i.m. with 5μg of LF and FB or a molar equivalent (6.5μg) of LFB. B5R (A) and L1R (B) specific IgG responses were determined by ELISA.
Figure 20  B5R is not recognized by B5R-specific antibodies in the context of the LFB trifusion.  B5R-specific plasma was generated by immunizing mice twice i.m. with B5R adsorbed on alum.  ELISA plates were coated with baculovirus-produced B5R or LFB. Wells were probed with the B5R-specific plasma and followed by a HRP-conjugated anti-mouse IgG antibody.
DISCUSSION

A major goal of these studies was to evaluate the efficacy of flagellin as an adjuvant with proteins that are poorly immunogenic in their recombinant forms. Our studies clearly demonstrate flagellin can be an effective adjuvant with weakly immunogenic antigens. Although administration of L1R, B5R, and flagellin promoted antigen-specific humoral responses, fusion proteins were clearly more potent. This was especially evident in the B5R-specific response. Even after 4 immunizations with the separate antigens 40% of mice did not respond to B5R (Figure 2). Alternatively, after 3 immunizations with the LF and FB fusion proteins all mice have B5R-specific IgG titers (Figure 8). This observation indicates that the mice have the full potential for a humoral response to B5R. One possible explanation for the low titers after immunization with separate antigens is a low frequency of B5R-specific CD4 T cells to aid in B cell activation and class switching. When the pox antigens are delivered in the form of fusion proteins, flagellin-specific CD4 T cells can activate B5R-specific B cells via linked recognition, thus overcoming a shortage of B5R-specific CD4 T cells.

Another factor that may play a role in the immunogenicity of a flagellin-antigen fusion protein is stability. Antigens which persist longer have a greater potential to be targeted for immune responses. It is possible that one of the benefits of flagellin fusion proteins is that by linking the poxvirus antigens to the highly stable flagellin molecule the stability of L1R and B5R is increased.

Even with the fusion proteins there was a requirement for at least three immunizations with LF and FB for protection against respiratory challenge with vaccinia
virus. Fogg et al. (36) found that four immunizations with recombinant poxvirus antigens were required to achieve high antibody titers and protection against challenge. In contrast to the more limited response to the recombinant poxvirus antigens, a single exposure to vaccinia virus elicits high titers of anti-L1R and B5R antibodies. At the most simple level, a sublethal dose of vaccinia virus may be more effective than immunization with a small number of recombinant antigens due to the availability of a dramatically larger number of potentially protective antigens during virus infection. However, it is also possible that there is some feature of antigens such as L1R and B5R in the context of the viral infection that cannot be replaced or overcome by flagellin or other adjuvants (30,36,37,46,49-51).

The duration of antigen availability represents another major difference between live virus vaccination and recombinant protein immunization. The persistence of antigen in the viral infection, as opposed to the limited availability of the flagellin fusion proteins following immunization provides a reasonable explanation for the difference in the level of the induced antibody response. Dryvax immunization results in the shedding of live vaccinia virus for 3-4 weeks (38,57). In contrast, flagellin administered i.m. is no longer detectable in the draining lymph nodes after 24 hours (Bates and Mizel, unpublished observations). In addition to antigen availability, the poxvirus-induced innate immune response may promote a more robust activation of dendritic cells and thus enhanced helper T cell activation and associated B cell responsiveness. Finally, it may be that the conformations of the recombinant L1R and B5R do not precisely mirror those of the same proteins in the context of a vaccinia virus infected cell.
In contrast to immunization with a sublethal dose of vaccinia virus, significant weight loss is observed in mice immunized with recombinant L1R and B5R. It is interesting to note that this weight loss in the LF + FB immunized mice occurred in the absence of other disease symptoms. In comparison to our protein-based vaccination strategy, recombinant viruses such as VSV (C. Lambeth et al., submitted for publication) and SV5 (K. Clark and G. D. Parks, unpublished observations) engineered to express L1R and B5R also promote protection in the presence of significant weight loss. In the case of SV5 and VSV, it may be that more limited activation of DC may be a more important factor in the reversible weight loss than antigen persistence or conformation.

Complement is known to be a part of the host response to vaccinia virus, especially in promoting lysis of the extracellular enveloped virus (EEV) (65,66). Clearance of EEV is very important as this form of the virus enters cells more rapidly than the intracellular mature virion (IMV) form (28), and it is released earlier in infection (before lysis of the host cell). In a recent study (13), Benhnia et. al. generated a series of monoclonal anti-B5R antibodies and screened them for their ability to protect mice from vaccinia virus. The only recovered monoclonal which conferred full protection at the administered doses was of the IgG2a isotype—an isotype in mice that is particularly efficient at activation of the classical complement pathway. Furthermore, Benhnia et. al. (13) demonstrated that depletion of complement reduced the protective effect of passive immunization with anti-B5R IgG2a. Our finding that the B5R-specific IgG2a response was significantly higher in mice that survived challenge with vaccinia virus is consistent with the findings of Benhnia et al. we found that 50% of the mice given the CVF treatment survived vaccinia virus challenge. There are several possible explanations for
this observation. First, it is possible that the return of complement levels in some of the depleted mice may be more rapid, thus providing these mice with sufficient complement to meet the needs of a protective response. Alternatively, other factors may contribute to the observed protection. LF +BF immunized mice generate multiple antigen-specific IgG isotypes. Although IgG1 is a less efficient activator of the classical complement pathway than IgG2a in mice (83), it is quite likely that sufficiently high titers of antigen-specific IgG1 may compensate for reduced inherent complement activating activity. Additionally, the LF + FB-induced IgG may promote clearance of virus by opsonization or direct neutralization.

One interesting observation was that in mice immunized with the fusion proteins B5R-specific IgG2a titers no longer correlated with survival as we had seen in mice immunized with separate pox antigens + flagellin (Figure 7). Of the mice challenged after 2 immunizations there was no significant difference in B5R-specific IgG2a titers between survivors and non-survivors (data not shown). This may be the result of flagellin fusion proteins inducing more broad activation of the immune system which would lessen the dependence on a single mechanism of protection. For example, very high IgG1 titers may be able to activate complement to a level that compensates for reduced IgG2a responses.

Despite the fact that i.m. immunization with fusion proteins generated greater antigen-specific IgG responses to poxvirus antigens than the separate proteins, three immunizations were still required for all of the mice to survive vaccinia virus challenge. The finding that three immunizations results in a higher level of vaccinia virus neutralizing activity than two immunizations (Figure 13), raises the possibility that
elevation of the neutralizing titer following a third immunization is a key event. Fogg et al. also observed an increase in neutralizing titer between two and three immunizations (37).

Although a mechanism for neutralization of vaccinia virus is not known, it has been hypothesized to be caused by steric hindrance (66). Steric hindrance could be achieved by antibodies of very high affinity or that interact with epitopes that are near or involved in virus-host cell interactions. With regard to antibody affinity, we found that the average avidity of mice immunized 3 times i.m. with LF and FB was 2.1 times higher than those immunized only twice (data not shown). It is also possible that the general response to neutralizing epitopes comes up more slowly, possibly due to a lower frequency of neutralizing epitope-specific B cells.

The avidity of an antibody response is refined in germinal centers where B cells interact with helper T cells to proliferate and undergo somatic hypermutation, affinity maturation, and isotype switching. The limited number of immune complexes presented on follicular dendritic cells create strict competition for antigen, therefore only the highest avidity clones can expand. There are several possible factors that could influence the B cell response. First, if the flagellin-poxvirus antigen fusion proteins possess only a limited ability to induce antigen-specific CD4+ T cell activation, then multiple rounds of immunization would be required to achieve a sufficient number of these cells to facilitate B cell activation and maturation. Thus the delay in the generation of high neutralization titers may be due to not only the limited frequency of B cells with specificity for neutralizing epitopes on L1R and B5R, but also a limited expansion of antigen-specific CD4+ T cells.
Our neutralization data may provide a guide to the minimal levels of neutralizing IgG required for survival. With average neutralization titers of ~300, some mice survive infection while others succumb. Mice that had mean neutralization titers ranging from 900-1300 all survived and showed little to no disease symptoms. However, mice in this range did lose weight at the peak of the infection, but this weight loss was reversible. When mice were immunized with sublethal doses of vaccinia virus the average neutralization titer was ~2700. These mice do not lose weight when challenged with high doses of vaccinia virus, thus exhibiting maximal protection. Thus it appears that a neutralizing titer between approximately 300 and 900 is required for survival, whereas a titer above 1300 is key for protection against weight loss.

Virus neutralization may only be part of the explanation for a difference in survival after two versus three immunizations. In a toxicology study in rabbits done according to currently accepted Good Laboratory Practices (Mizel, unpublished observations), it was observed that two immunizations with 100, 250, or 500 μg of a flagellin fusion containing the *Y. pestis* antigens F1 and V did not induce any significant changes in blood chemistry. However, after a third immunization with 250 or 500 μg, there was a significant increase in several liver enzymes indicating hepatic activation. One result of liver activation is the release of the complement activating and opsonic acute phase reactant, C-reactive protein. However, the circulating levels of CRP in the blood did not change following immunization with flagellin.

Although flagellin did not increase circulating levels of CRP (Figure 14A), it might have triggered an increase in neutrophils available for the clearance of antibody-coated vaccinia virus (Fig. 14B). In this regard, a study comparing motile (flagellated)
and non-motile (non-flagellated) strains of *Pseudomonas aeruginosa* revealed that only the strain expressing flagellin induced GM-CSF production in primary human lung cells (19). After 7 days blood neutrophil levels were all equal in immunized and unimmunized mice (data not shown), so it is unlikely that increased numbers of neutrophils (as a result of a third immunization) are contributing to protection since mice were challenged with vaccinia virus 3 weeks after the final immunization.

It remains that the observation that neutrophil levels are increased immediately after exposure to a pathogen-associated molecular pattern (PAMP) could enhance clearance of a pathogen, even though it may not play a role in protection following vaccination. However, it is possible that exposure to the virus acted as another danger signal and itself induced an enhanced response as a result of the “priming” the host received during vaccination.

Multiple exposures to danger signals such as flagellin and other PAMPs would signal to the host that the efforts to clear the pathogen have failed. It seems logical that individuals that possess enhanced immune responsiveness when frequently re-exposed to a pathogen would have an evolutionary advantage. It would be of great interest to determine if multiple PAMPs can promote this enhanced response to infectious agents, and whether the types of responses would be tailored to the type of pathogen from which the PAMP was derived.

Take together, these results establish that flagellin can promote robust responses against weakly immunogenic poxvirus antigens. Although weight loss is observed, the immunized mice do not exhibit other signs of disease and survival challenge. It may be
that the overall effectiveness of a flagellin/poxvirus fusion protein vaccine may be enhanced by inclusion of additional target antigen/flagellin fusion proteins.

The results of an increasing number of studies clearly demonstrate that flagellin is a potent adjuvant when delivered intranasally, intramuscularly, or intradermally (11,20,48,52,53,62,69,70,75,98). A particular strength of flagellin is that its adjuvant properties are not inhibited if the host has pre-existing immunity to flagellin (10). Flagellin elicits robust antigen-specific humoral responses without promoting deleterious antigen-specific IgE responses or serious adverse inflammation (47,48). Flagellin is especially potent at promoting antigen-specific responses when the antigen is genetically linked to flagellin (7,11,12,48,52,53,56,62,69,70,75,105). Our studies reinforce this concept. Administration of the LF and FB fusion proteins decreased the required molar doses, raised total antigen-specific titers, increased the number of mice that seroconverted, and decreased variability among animals. Similar trends were seen by Weimer et al. in the context of a flagellin-based fusion protein vaccine against Pseudomonas aeruginosa (105).

Another benefit of flagellin is that it possesses substantial plasticity as an antigen carrier. Depending on the antigen, target sequences can be inserted at the amino or carboxy termini as well as within the hypervariable region without loss of TLR5 signaling and adjuvant activity (Figures 1 and 14 and (7,10-12,20,52,53,56,60,62,70,75,105)). Furthermore, these flagellin fusion proteins can be produced in bacteria and purified in large amounts under conditions that are compatible with current Good Manufacturing Practices (75).
The purpose of our studies with flagellin and poxvirus antigens was to “test the limits” of flagellin as an adjuvant by using L1R and B5R, two antigens that are poorly immunogenic in their recombinant forms. Our studies clearly demonstrate that there are limitations on the carrier function of flagellin. Although TLR5 signaling activity was preserved in all of the flagellin fusion proteins used in this study, in some instances (FL and LFB; Figure 14) the immunogenicity of the antigen was lost. There are two possible explanations for the lack of IgG that recognized the native antigen. In these fusion proteins, there may have been a deviation from the normal conformation of L1R in FL or LFB. This could be from changes in folding or the pattern of glycosylation. An alternative explanation is that a decrease in accessibility of epitopes to B cell receptors. Since this problem is antigen-specific, it is essential that for each new flagellin-antigen fusion protein, multiple insertion sites on flagellin need to be evaluated to obtain a fusion that results in retention of TLR5 signaling activity as well as the production of antibodies with specificity for the native antigen.

The use of flagellin as a carrier for weakly immunogenic proteins may be limited by the potential for some form of antigenic competition (Figures 17 and 18). The limited competition did not appear to have a negative effect on a vaccine formulation containing only two fusion proteins. However, a problem may arise if a vaccine contains more than two flagellin fusion proteins. There are many vaccines, such as those targeting *Streptococcus pneumoniae* and *Haemophilus influenzae*, which are multivalent to provide protection against a broad range of clinical isolates. For these pathogens, flagellin fusion proteins might be limited in their efficacy unless successful multi-antigen fusion proteins or conjugates could be generated.
While we were unable to generate a flagellin-pox antigen trifusion protein that retained immunogenicity of all components, such fusions have been made against other targets (48,75,105). Again, this is an antigen-driven limitation. Each future vaccine strategy involving flagellin-antigen fusion proteins will require testing of multiple insertion sites and insertion order when generating fusions involving 3 or more proteins.

In summary, flagellin greatly enhances protective humoral immune responses against weakly immunogenic proteins. The most efficient way to use flagellin with the poxvirus antigens L1R and B5R is in the form of single flagellin-antigen fusion proteins that are administered intramuscularly.
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Abstracts